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BASIC SCIENCE

Primary cell culture of meningothelial cells—a new model to study the arachnoid in glaucomatous optic neuropathy

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Abstract
Background In a previous report, we found that the occurrence and amount of meningothelial cell nests in the subarachnoid space are significantly increased in glaucomatous optic nerves compared to normals. In order to allow research into the role of meningothelial cells during diseases of the optic nerve, an in vitro model is necessary. For this purpose, we developed a culture method for porcine meningothelial cells from the arachnoid layer covering the optic nerve.

Methods Meningothelial cells were scraped from the arachnoid layer of porcine optic nerves and cultured for 2–3 weeks until the cells formed a monolayer. To eliminate contaminating fibroblasts from the culture, cells were negatively selected using magnetic anti-fibroblast beads after the first passage. Cells were detached using 0.05% Trypsin-EDTA, incubated with anti-fibroblast beads, separated using a magnetic column and the flow-through was collected. The purified primary meningothelial cells were characterized by electron microscopy and immunocytochemistry using anti-glial fibrillary acidic protein (GFAP) and anti-keratan sulfate antibodies.
Results Primary cells grew out after dissection and formed a monolayer within 2–3 weeks, which was composed of two morphologically different cell types, flattened cells with round nuclei and fibroblast-like cells with long processes. The fibroblast-like cells in the culture could be labelled and selected using anti-fibroblast microbeads. The second cell type did not bind to the anti-fibroblast beads, and upon immunocytochemistry showed a marked expression of both GFAP and keratan sulphate. In addition, examination of these cells by electron microscopy revealed morphological characteristics of meningothelial cells, including hemidesmosomes and cytoplasmatic filaments.
Conclusions The technique described in this paper for the primary culture of meningothelial cells from the subarachnoid space of the optic nerve and using magnetic beads for the removal of fibroblasts is effective in obtaining a highly enriched meningothelial cell culture.

Keywords Meningothelial cell · Fibroblast · Subarachnoid space · Cell culture · Optic nerve · Glaucoma · Arachnoid

The authors have full control of all primary data, and they agree to allow Graefe's Archive for Clinical and Experimental Ophthalmology to review their data upon request.

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Introduction

The optic nerve is a white matter tract of the central nervous system that connects the retinal axons to the brain. It is enveloped by the meninges throughout its intraorbital and intracanalicular course. The meningeal sheath of the optic nerve has the same lamellar structure as the meninges of the brain, the pia mater, the arachnoid and the dura mater. The inner layer of the arachnoid is covered with meningothelial cells. A variety of trabecules and septae covered with meningothelial cells transverse the subarachnoid space between the arachnoid and the pia. Located between the dura and the arachnoid mater is the subdural space [1, 2].The subdural and the subarachnoid space of the optic nerve are continuous with those of the brain.

The subarachnoid space and the subdural space are occupied by the cerebrospinal fluid (CSF). CSF is mainly produced by the choroid plexus epithelium and the ependymal cells of the ventricular system, from where it flows into cisterns and the subarachnoid spaces, including the subarachnoid space of the optic nerve [3]. CSF is generally thought to communicate freely among the different CSF spaces and to have a homogeneous pressure throughout the brain and optic nerve. However, recent anatomical studies demonstrated that a complex architecture of arachnoid trabeculae, pillars, and septa in the subarachnoid space of the optic nerve might strongly influence CSF dynamics in the subarachnoid space via a dynamic valve function which might lead to a pressure gradient along the optic nerve [2, 4].

Meningothelial cells that cover the meninges of the optic nerve build a fluid barrier that prevents the outflow of CSF. Aging, and disorders such as meningitis, can influence the anatomical structure of the optic nerve sheath. A recent study demonstrated an age-dependent increase of meningothelial cell proliferation, causing the formation of cell nests, which eventually hyalinize and calcify [5]. A significant increase in occurrence and size of those meningothelial cell nests in the subarachnoid space has been observed in glaucoma compared to healthy optic nerves. The function is yet not known; however, a secondary reaction of the meningothelial cells to axon loss during glaucoma can be assumed [1]. In addition, impairment of meningothelial cell layer function might result in the failed clearance of biologically active molecules or waste products from the subdural and subarachnoidal space, and this might contribute to optic nerve diseases such as progressive anterior and posterior ischemic optic neuropathy, or optic nerve sheath compartment syndrome and even glaucoma [4, 6–8].

As the major cell population covering the orbital optic nerve sheath, the possible involvement of meningothelial cells in optic nerve diseases warrants further investigation. The culture of primary meningothelial cells from the optic nerve sheath makes it possible for the first time to study the response of those cells to various physiological and pathological conditions, and a more detailed understanding of their function during health and disease might improve clinical intervention for optic nerve disorders.

Materials and methods

Dissection and cell culture

Porcine eyes were obtained from the local slaughter house within 4 h of death. Eyes were transported to the laboratory in Krebs-Ringer solution, and rinsed with sterile phosphate buffered saline (PBS, Sigma, Germany) containing 10,000 U/ml penicillin and 10,000 ug/ml streptomycin (Sigma, Germany). Tissue and muscles around the eye and the optic nerve were removed (Fig. 1a). The dura-arachnoid sheath was freed from the optic nerve by using two tooth-forceps, one for fixating the optic nerve, and the other for tearing the dura-arachnoid sheath towards the globe while keeping the subarachnoid side upward (Fig. 1b). The dissected layer was transferred to a sterile, collagen precoated, 35-mm cell culture dish (Fig. 1c) containing 1 ml Dulbecco's modified Eagle's medium (DMEM, Gibco, Germany) supplemented with 10% fetal bovine serum (FBS, Sigma, Germany) and PSA (10,000 U/ml penicillin, 10,000 ug/ml streptomycin, 25 ug/ml amphotericin B). Collagen-coating was done with 0.1% Bornstein and Traub Type I collagen in 0.1 M acetic acid (Sigma, C8919) by covering the plates with collagen followed by 1 hour air-drying. With the subarachnoid layer facing upwards, superficial cells were gently scratched from the subarachnoid side into the medium using a blade (Fig. 1d). Cells were incubated at 37°C in a 95% air/5% CO_2 atmosphere. The growth medium was changed twice a week.

Separation of meningothelial cells from contaminating fibroblasts

After outgrowth, cells were washed with PBS, trypsinized (0.05% Trypson-EDTA) for 2 min, and centrifuged at 1100 rpm for 3 min at room temperature. The supernatant was aspirated and the cell pellet was resuspended in PBS buffer (0.5% BSA, 2 mM EDTA, pH 7.2), anti-fibroblast microbeads (Miltenyi Biotec GmbH, Germany) (20 ul/10^7 cells) were added and incubated for 30 min at room temperature. Cells were washed with PBS buffer, centrifuged at 100×g for 10 min, and were resuspended in PBS buffer. To eliminate fibroblasts, 0.5 ml cell suspension were

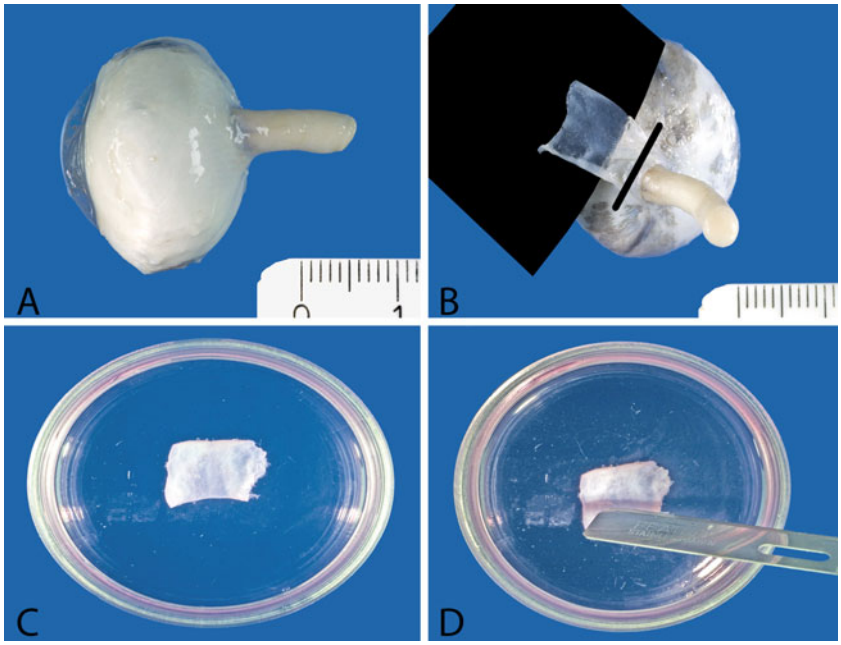

Fig. 1 **a** Porcine eye with anterior part of the optic nerve surrounded by the optic nerve sheath. **b** Dura and arachnoid layers were cut and torn toward the globe by keeping the subarachnoid side upward. **c** The dissected layer was transferred into a dish with culture medium. **d** Superficial cells were gently scraped from subarachnoid side into the medium by using a blade

applied onto MACS column (Miltenyi Biotec GmbH, Germany); the cells flow through column were collected as meningothelial cells and analyzed for the presence of meningothelial cells by immunochemistry and electron microscopy. Fibroblasts were recovered by eluting the magnetically labeled cells from the column with a plunger.

Immunocytochemistry

After magnetic separation, the two cell populations were spun onto slides using a Cytospin III centrifuge (Shandon, Pittsburgh, PA, USA). The cells were fixed with 4% carbodiimide in PBS, incubated with GFAP antibody (Santa Cruz, CA, USA) that diluted to 1:100 in PBS. The antigen was visualized using goat anti-mouse IgG conjugated to horseradish peroxidase and 3,3-diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin, dehydrated and mounted. Incubation without primary antibodies served as negative control.

After separation, meningothelial cells were grown on coverslips, fixed with acetone and methanol for 3 min, permeabilized with 0.1% Triton X-100+1% BSA/FCS in PBS for 15 min at room temperature, rinsed three times with PBS and non-specific binding sites were blocked with 1% BSA in PBS/0.1% Triton X-100 for 1 h. The cells were incubated with anti-keratan sulphate antibodies (AbD Serotec, USA) for 2 h at room temperature; the concentration of antibody was diluted to a ratio of 1:100, and then washed three times with PBS. The primary antibody was visualized using Alexa Fluor 488-labeled anti-mouse secondary antibodies (Invitrogen, USA). Cells were counterstained with DAPI for 3 min, mounted on glass slides, and observed with a fluorescence microscope (Olympus B×51, Japan).

Ultrastructural examination by electron microscopy

After magnetic separation, 4 to 5×10^6 meningothelial cells were pelleted and fixed for 180 min with cold 3% glutaraldehyde in 0.2 M sodium cacodylate phosphate buffer. After a brief rinse with PBS buffer, pellets were postfixed for 1 hr with cold 1% osmium tetroxide in phosphate buffer, pH 7.3. The pellets were cut into fragments. Both dehydration in ethanol and propylene oxide and embedding in Epon were done by routine procedures; 1.5 µm sections were mounted on slide and stained with toluidine blue. After that, 85 nm sections mounted on uncoated grids and stained with uranyl and lead were imaged using a FEI Morgagni electron microscope.

Results

Primary cell culture from the subarachnoid layer of porcine optic nerves

Cells grew out after dissection and formed a monolayer within 2–3 weeks, which was composed of two different cell types, flattened cells with round nuclei and fibroblast-like cells with long processes (Fig. 2a).

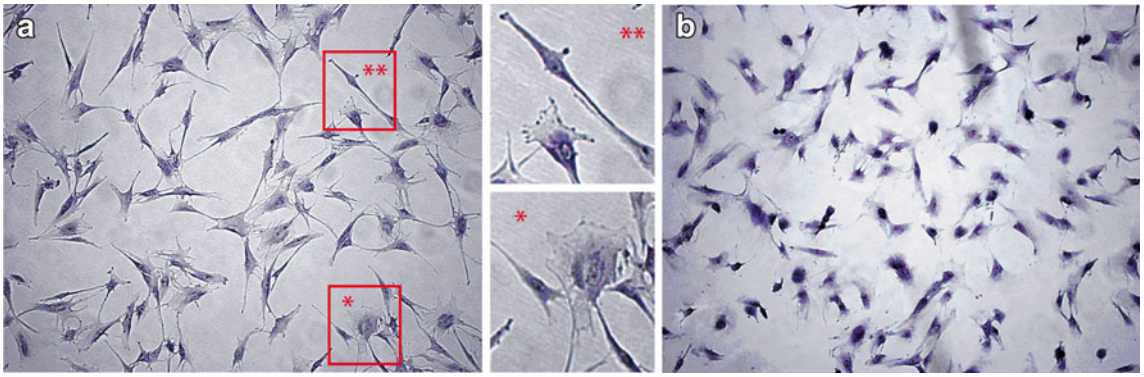

Fig. 2 **a** Micrograph of primary cultured cells shows two different cell types, flattened cells with round nuclei (*) and cells with long processes (**) (fibroblast-like). **b** Meningothelial cells after separation with anti-fibroblast microbeads (×10). The results are shown for a representative cell culture out of four independent primary cell preparations

Purification of meningothelial cells with magnetic anti-fibroblast microbeads

Cells were separated by magnetic fibroblast microbeads after primary cells reached confluency; positive and negative cells were collected respectively and continued to be cultured. Positive cells labelled with fibroblast microbeads showed obvious enlongated fibroblast-like morphology; negative cells against fibroblast bead presented flattened cell type (Fig. 2b).

Characterization of meningothelial cells with GFAP and keratan sulphate antibodies

Based on the observation of GFAP staining at the optic nerve, we found that not only astrocyte expressed GFAP, but meningothelial cells also exhibited positive immunostaining for GFAP. We used GFAP as well as keratan sulphate antibody as markers for identifying meningothelial cells. The result revealed strong expression of meningothelial cells for both GFAP and keratan sulphate antibodies (Fig. 3a, b), while fibroblasts stained negative for GFAP (Fig. 3c). To establish the purity of the meningothelial cell culture, cells after six passages were stained with anti-GFAP antibodies and counterstained using DAPI. Counting revealed less then 1% GFAP-negative cells (99.3% GFAP positive, $n=3$) confirming a pure meningothelial cell culture.

Morphological analysis of meningothelial cells by electron microscopy

The ultrastructural analysis revealed cells with unlabelled anti-fibroblast microbeads which contained oval nuclei (Fig. 4a) and showed slight membrane irregularity. Desmosomes were found between the cells (Fig. 4b). The cytoplasm contained filaments arranged in aggregates (Fig. 4c).

Discussion

In order to establish a method for the culture of primary meningothelial cells, we used a cut and tear method to separate the arachnoid layer from the optic nerve. We found this method to be superior to mechanical dissection of the arachnoid layer in terms of speed and the yield of meningothelial cells.

Meningothelial cells are the most common cell type, covering both sides of the arachnoid layer; however, there are fibroblasts present in the arachnoid trabeculae in the form of a very thin connective tissue. Therefore, they are always part of the primary culture of meningothelial cells, and the removal of those contaminating fibroblasts is crucial to get pure meningothelial cells. The use of magnetic anti-fibroblast microbeads proved useful to separate out fibroblasts and purify meningothelial cells for further cultivation.

For identification of the purified meningothelial cells, we relied on a previous observation described by Hogan [5]; in contrast to the contaminating fibroblasts, the morphology of the meningothelial cells appeared to be flattened. Furthermore, immunocytochemical analysis identified the negatively selected cells as meningothelial cells. We found that meningothelial cells stained positive for GFAP, which is in accordance with our findings in post-mortem optic nerves (our unpublished observation) and the positive GFAP staining of ependymal cells as shown by Kivelä [9]. In addition to GFAP, keratan sulfate was previously reported to be expressed in meningothelial cells [10] and, as expected, staining of the purified meningothelial cells revealed the presence of this marker protein in our cultured

primary cells. Furthermore, the ultrastructural features of the primary cells we cultivated are consistent with meningothelial cells, which presented with fine filaments extending throughout the cytoplasm, frequently joined by desmosomes with other cells.

Recent studies have brought new understanding to the pathogenesis of optic nerve diseases, in which the generally accepted concept that the CSF pressure and composition are homogeneous within small limits throughout all CSF compartments has been challenged by evidence suggesting that the subarachnoid space of the optic nerve can become separated from other CSF spaces in the wake of optic nerve disease. Compartmentation of the subarachnoid space of the optic nerve might result in an imbalanced composition of the CSF due to impaired removal of waste products [11, 12]. This is thought to contribute to the development of optic nerve sheath compartment syndromes, and might be related to progressive anterior and posterior ischemic optic neuropathy or chronic open-angle and normal-tension glaucoma [10, 13–15]. Meningothelial cells are the elemental cells lining the inner surface of the dura mater, both side of arachnoid layer, and the outer surface of pia mater, and are therefore in direct contact to the CSF. They perform an essential role in keeping the CSF barrier intact, and it is conceivable that any ailment of meningothelial cells such as inflammation, ischemia or and hypoxia possibly results in the impairment of the CSF composition. The significantly increased presence of the meningothelial cell nests in the subarachnoid space in glaucoma has drawn our attention and interest to further investigate the pathophysiological role of meningothelial cells in optic nerve diseases. Taken together, the primary culture of meningothelial cells presented here is the first step in studying those cells in vitro, and might prove useful in the future to unravel the etiology of optic nerve diseases.

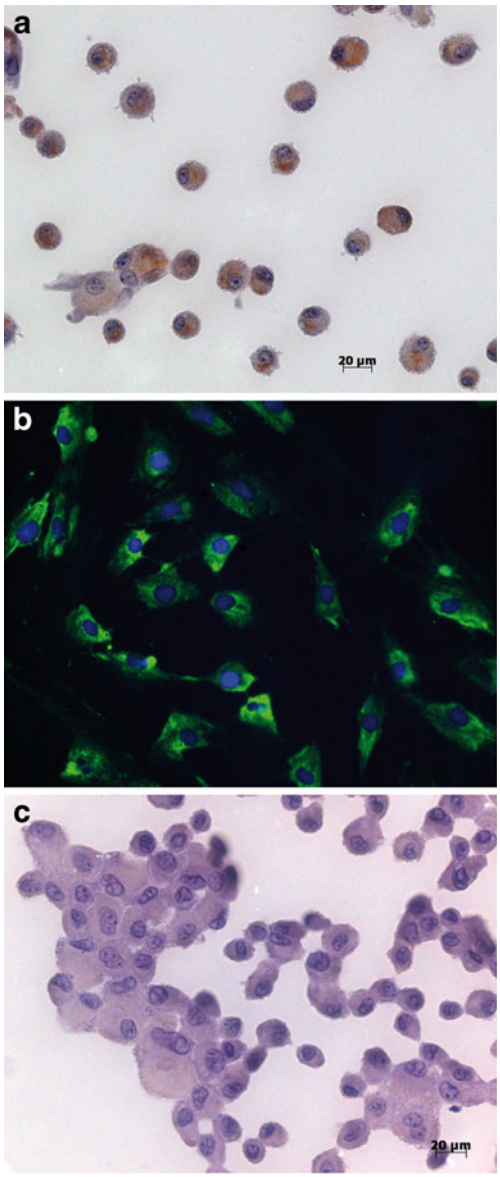

Fig. 3 **a** Positive immunostaining of meningothelial cells for GFAP antibody after the separation with anti-fibroblast microbeads. **b** Marked immunostaining of meningothelial cells for keratan sulphate antibody after separation with microbeads (×40). **c** Anti-fibroblast beads selected cells stained negative for GFAP using anti-GFPA antibodies, making GFAP a good marker to distinguish between meningothelial cells and contaminating fibroblasts

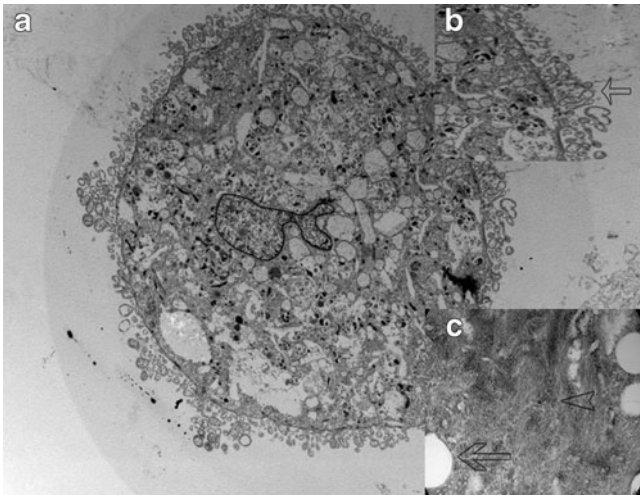

Fig. 4 Electron micrograph of meningothelial cells (**a**) with ovoid nuclei. Ultrastructural features of hemidemosomes (*arrows*) (**b**), filaments (*arrowhead*), and lipid vacuoles (*arrow*) (**c**) of meningothelial cells

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